

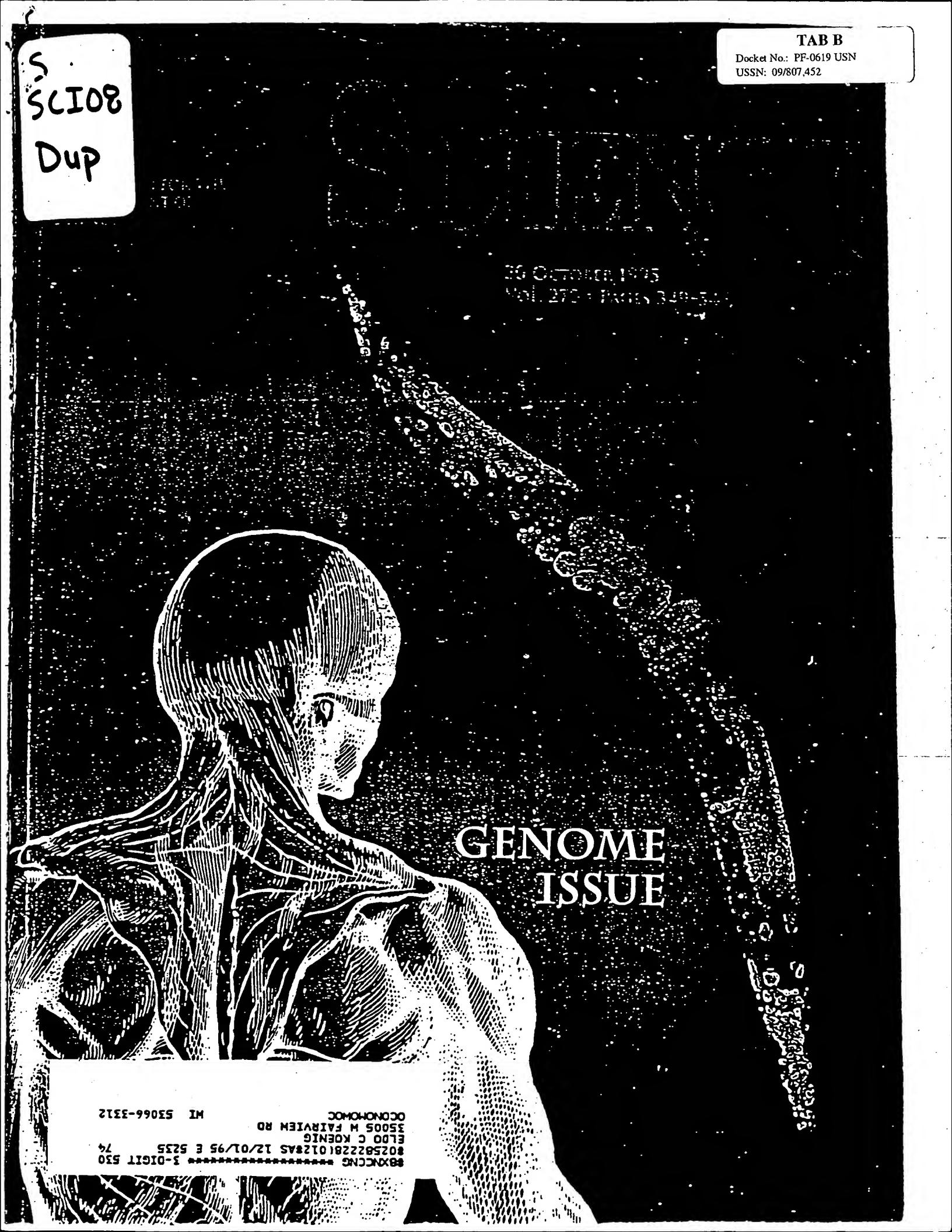
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# GENOME ISSUE

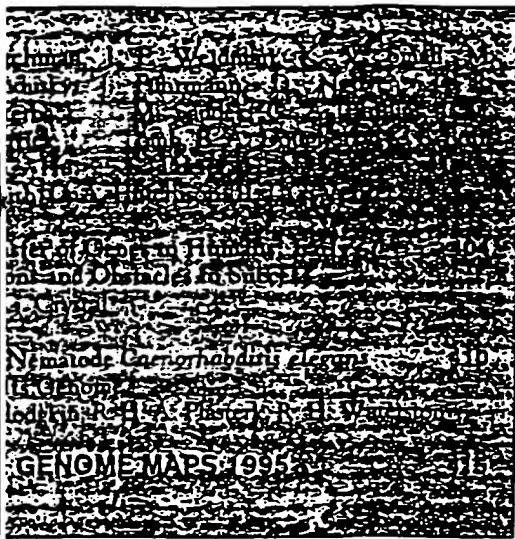
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## COVER

The Genome Project adds a new dimension to questions of gene expression in humans and model systems. A report on page 415 summarizes progress in the *C. elegans* Genome Project and indicates some ways information about sequences can be used.

News stories, Articles, Perspectives, Policy Forums, and Reports focus on technological developments, clinical applications, and ethical concerns resulting from the burgeoning of genomic information. [C. elegans image: F. Maduro and D. Pilgrim, University of Alberta]



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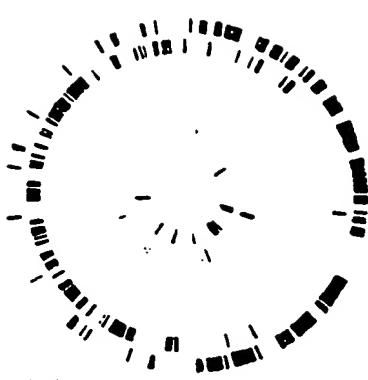
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Ad1p sequence following Ser<sup>208</sup> and occurs within the domain of Ad1p that shows homology with hIDE (14). To delete the complete STE23 sequence and create the ste23Δ::URA3 mutation, polymerase chain reaction (PCR) primers (5'-TCGGAAGACCTCAT-TCTTGCATTTGATATTGCTC-TGTAGATTG-TACTGAGAGTGAC-3'; and 5'-GCTACAAACAGC-GTCGACTTGAATGCCCGACATCTCGACTGT-GCGGTATTCACACCG-3') were used to amplify the URA3 sequence of pRS316, and the reaction product was transformed into yeast for one-step gene replacement [R. Rothstein, *Methods Enzymol.* 184, 281 (1991)]. To create the ad1Δ::LEU2 mutation contained on p114, a 5.0-kb Sal I fragment from pAXL1 was cloned into pUC19, and an internal 4.0-kb Hpa I-Xba I fragment was replaced with a LEU2 fragment. To construct the ste23Δ::LEU2 allele (a deletion corresponding to 831 amino acids) carried on p153, a LEU2 fragment was used to replace the 2.8-kb Pml I-Ecl136 II fragment of STE23, which occurs within a 6.2-kb Hind III-Bgl II genomic fragment carried on pSP72 (Promega). To create YEpmfA1, a 1.6-kb Barn HI fragment containing MFA1 from pKK16 (K. Kuchler, R. E. Stern, J. Thomer, *EMBO J.* 8, 3973 (1989)), was ligated into the Barn HI site of YEpmfA1 (J. E. Hill, A. M. Myers, T. J. Koerner, A. Tzagoloff, *Yeast* 2, 163 (1986)).

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- G. F. Sprague Jr., *Methods Enzymol.* 194, 77 (1991).
- Single-letter abbreviations for the amino acid residues are as follows: A: Ala; C: Cys; D: Asp; E: Glu; F: Phe; G: Gly; H: His; I: Ile; K: Lys; L: Leu; M: Met; N: Asn; P: Pro; Q: Gin; R: Arg; S: Ser; T: Thr; V: Val; W: Trp; and Y: Tyr.
- A W303 1A derivative, SY2625 (MATα ura3-1 leu2-3, 112 trp1-1 ade2-1 can1-100 sst1 Δ mfa2Δ; FUS1- lacZ his3Δ::FUS1-HIS3), was the parent strain for the mutant search. SY2625 derivatives for the mating assays, secreted pheromone assays, and the pulse-chase experiments included the following strains: Y149 (ste22-1), Y115 (mfa1Δ::LEU2), Y142 (ad1Δ::URA3), Y173 (ad1Δ::LEU2), Y220 (ad1Δ::URA3 ste23Δ::URA3), Y221 (ste23Δ::URA3), Y231 (ad1Δ::LEU2 ste23Δ::LEU2), and Y233 (ste23Δ::LEU2). MATα derivatives of SY2625 included the following strains: Y199 (SY2625 made MATα), Y278 (ste22-1), Y195 (mfa1Δ::LEU2), Y196 (ad1Δ::LEU2), and Y197 (ad1Δ::URA3). The EG123 (MATα leu2 ura3 trp1 can1 his4) genetic background was used to create a set of strains for analysis of bud site selection. EG123 derivatives included the following strains: Y175 (ad1Δ::LEU2), Y223 (ad1Δ::URA3), Y234 (ste23Δ::LEU2), and Y272 (ad1Δ::LEU2 ste23Δ::LEU2). MATα derivatives of EG123 included the following strains: Y214 (EG123 made MATα), and Y293 (ad1Δ::LEU2). All strains were generated by means of standard genetic or molecular methods involving the appropriate constructs (23). In particular, the ad1Δ::ste23 double mutant strains were created by crossing of the appropriate MATα ste23 and MATα ad1Δ mutants, followed by sporulation of the resultant diploid and isolation of the double mutant from nonparental di-type tetrads. Gene disruptions were confirmed with either PCR or Southern (DNA) analysis.
- p129 is a YEpmf32 (J. E. Hill, A. M. Myers, T. J. Koerner, A. Tzagoloff, *Yeast* 2, 163 (1986)) plasmid containing a 5.5-kb Sal I fragment of pAXL1, p151 was derived from p129 by insertion of a linker at the Bgl II site within AXL1, which led to an in-frame insertion of the hemagglutinin (HA) epitope (DQYPYDVPDYA) (29) between amino acids 854 and 855 of the AXL1 prod-

uct. pC225 is a pRS+ (Stratagene) plasmid containing a 0.5-kb Barn HI-Sst I fragment from pAXL1. Substitution mutations of the proposed active site of Ad1p were created with the use of pC225 and site-specific mutagenesis involving appropriate synthetic oligonucleotides (ad1Δ-H684, 5'-GTGCTCACAAAGCGT-GCCAAACGGC-3'; ad1-E71A, 5'-AAGAATCAT-GTGCGCACAAAGGTGCGC-3'; and ad1-E71D, 5'-AAGAATCATGTGATCACAAAGGTGCGC-3'). The mutations were confirmed by sequence analysis. After mutagenesis, the 0.4-kb Barn HI-Msc I fragment from the mutagenized pC225 plasmids was transferred into pAXL1 to create a set of pRS316 plasmids carrying different AXL1 alleles, p124 (ad1Δ-H684), p130 (ad1-E71A), and p132 (ad1-E71D). Similarly, a set of HA-tagged alleles carried on YEpmf32 were created after replacement of the p151 Barn HI-Msc I fragment, to generate p161 (ad1-E71A), p162 (ad1-

- H684), and p163 (ad1-E71D).
- We thank J. Becker and S. Michaels for providing α-factor antibodies; S. Michaels for discussing unpublished results and helping with the pulse-chase experiments; J. Brown, J. Chant, and S. Sanders for their input concerning bud site selection experiments; M. Raymond, F. Tamino, and M. Whitewell for plasmids; M. Marrs for providing the STE23 genomic fragment; and H. Bussey, J. Brown, N. Davis, T. Favero, C. de Hoog, and S. Kim for comments on the manuscript. Supported by a grant to C.B. from the Natural Sciences and Engineering Research Council of Canada. Support for M.N.A. was from a California Tobacco-Related Disease Research Program postdoctoral fellowship (4FT-0083).

22 June 1995; accepted 21 August 1995

## Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA Microarray

Mark Schena,\* Dari Shalon,\*† Ronald W. Davis, Patrick O. Brown‡

A high-capacity system was developed to monitor the expression of many genes in parallel. Microarrays prepared by high-speed robotic printing of complementary DNAs on glass were used for quantitative expression measurements of the corresponding genes. Because of the small format and high density of the arrays, hybridization volumes of 2 microliters could be used that enabled detection of rare transcripts in probe mixtures derived from 2 micrograms of total cellular messenger RNA. Differential expression measurements of 45 *Arabidopsis* genes were made by means of simultaneous, two-color fluorescence hybridization.

The temporal, developmental, topographical, histological, and physiological patterns in which a gene is expressed provide clues to its biological role. The large and expanding database of complementary DNA (cDNA) sequences from many organisms (1) presents the opportunity of defining these patterns at the level of the whole genome.

For these studies, we used the small flowering plant *Arabidopsis thaliana* as a model organism. *Arabidopsis* possesses many advantages for gene expression analysis, including the fact that it has the smallest genome of any higher eukaryote examined to date (2). Forty-five cloned *Arabidopsis* cDNAs (Table 1), including 14 complete sequences and 31 expressed sequence tags (ESTs), were used as gene-specific targets. We obtained the ESTs by selecting cDNA clones at random from an *Arabidopsis* cDNA library. Sequence analysis revealed that 28 of the 31 ESTs matched sequences

in the database (Table 1). Three additional cDNAs from other organisms served as controls in the experiments.

The 48 cDNAs, averaging ~1.0 kb, were amplified with the polymerase chain reaction (PCR) and deposited into individual wells of a 96-well microtiter plate. Each sample was duplicated in two adjacent wells to allow the reproducibility of the arraying and hybridization process to be tested. Samples from the microtiter plate were printed onto glass microscope slides in an area measuring 3.5 mm by 5.5 mm with the use of a high-speed arraying machine (3). The arrays were processed by chemical and heat treatment to attach the DNA sequences to the glass surface and denature them (3). Three arrays, printed in a single lot, were used for the experiments here. A single microtiter plate of PCR products provides sufficient material to print at least 500 arrays.

Fluorescent probes were prepared from total *Arabidopsis* mRNA (4) by a single round of reverse transcription (5). The *Arabidopsis* mRNA was supplemented with human acetylcholine receptor (AChR) mRNA at a dilution of 1:10,000 (w/w) before cDNA synthesis, to provide an internal standard for calibration (5). The resulting fluorescently labeled cDNA mixture was hybridized to an array at high stringency (6) and scanned

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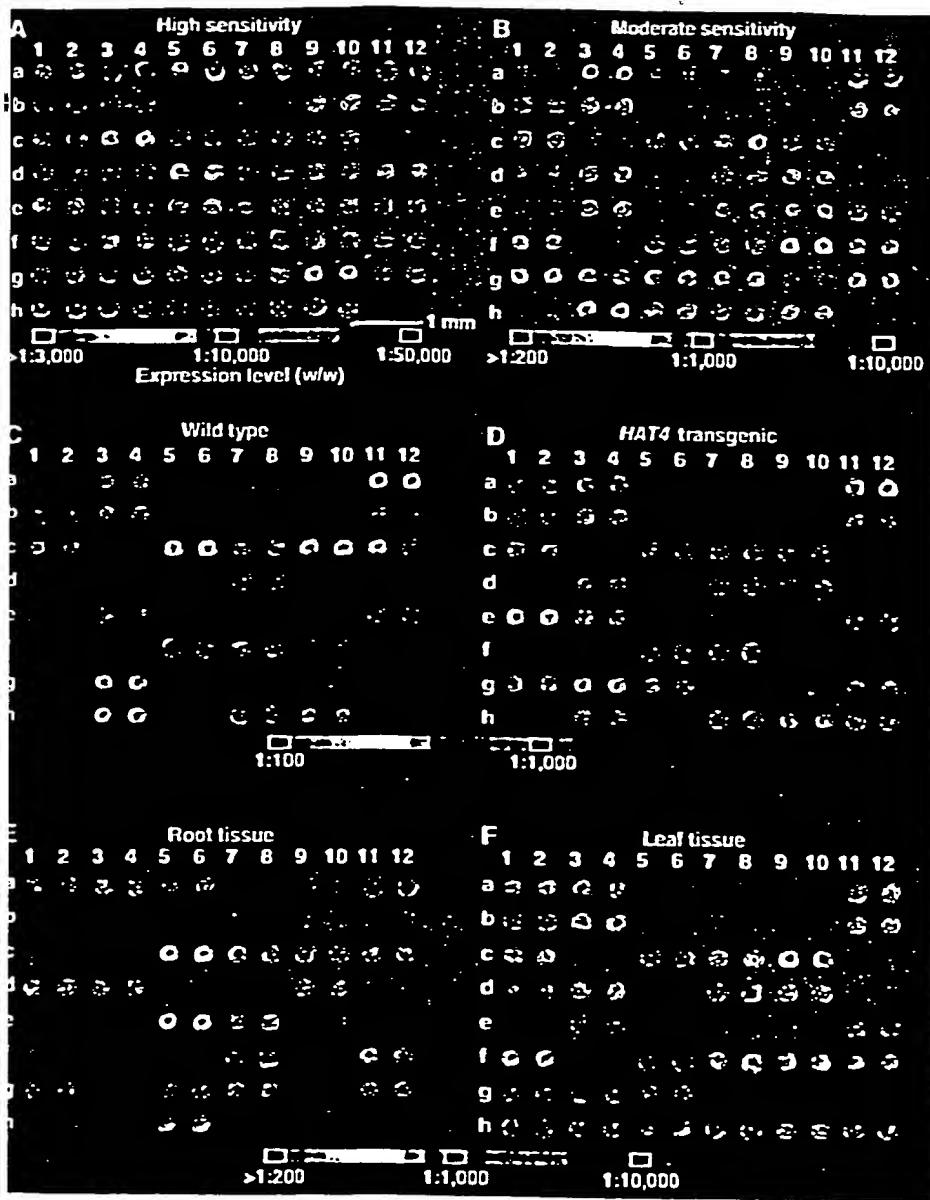
h a laser (3). A high-sensitivity scan gave signals that saturated the detector at nearly all of the *Arabidopsis* target sites (Fig. 1A). Calibration relative to the AChR mRNA standard (Fig. 1A) established a sensitivity limit of ~1:50,000. No detectable hybridization was observed to either the rat glucocorticoid receptor (Fig. 1A) or the yeast TRP4 (Fig. 1A) targets even at the highest scanning sensitivity. A moderate-sensitivity scan

of the same array allowed linear detection of the more abundant transcripts (Fig. 1B). Quantitation of both scans revealed a range of expression levels spanning three orders of magnitude for the 45 genes tested (Table 2). RNA blots (7) for several genes (Fig. 2) corroborated the expression levels measured with the microarray to within a factor of 5 (Table 2).

Differential gene expression was investi-

gated with a simultaneous, two-color hybridization scheme, which served to minimize experimental variation inherent in the comparison of independent hybridizations. Fluorescent probes were prepared from two mRNA sources with the use of reverse transcriptase in the presence of fluorescein- and lissamine-labeled nucleotide analogs, respectively (5). The two probes were then mixed together in equal proportions, hybridized to a single array, and scanned separately for fluorescein and lissamine emission after independent excitation of the two fluorophores (3).

To test whether overexpression of a single gene could be detected in a pool of total *Arabidopsis* mRNA, we used a microarray to analyze a transgenic line overexpressing the single transcription factor HAT4 (8). Fluorescent probes representing mRNA from wild-type and HAT4-transgenic plants were labeled with fluorescein and lissamine, respectively; the two probes were then mixed and hybridized to a single array. An intense hybridization signal was observed at the position of the HAT4 cDNA in the lissamine-specific scan (Fig. 1D), but not in the fluorescein-specific scan of the same array (Fig. 1C). Calibration with AChR mRNA added to the fluorescein and lissamine cDNA synthesis reactions at dilutions of 1:10,000 (Fig. 1C) and 1:100 (Fig. 1D), respectively, revealed a 50-fold elevation of HAT4 mRNA in the transgenic line relative to its abundance in wild-type plants (Table 2). This magnitude of HAT4 overexpression matched that inferred from the Northern (RNA) analysis within a factor of 2 (Fig. 2 and Table 2). Expression of all the other genes monitored on the array differed by less than a factor of 5 between HAT4-transgenic and wild-type plants (Fig. 1, C



1. Gene expression monitored with the use of cDNA microarrays. Fluorescent scans represented in pseudocolor correspond to hybridization intensities. Color bars were calibrated from the signal obtained with the use of known concentrations of human AChR mRNA in independent experiments. Numbers and letters on the axes mark the position of each cDNA. (A) High-sensitivity fluorescein scan after hybridization with fluorescein-labeled cDNA derived from wild-type plants. (B) Same array as in (A) but scanned at moderate sensitivity. (C and D) A single array was probed with a 1:1 mixture of fluorescein-labeled cDNA from wild-type plants and lissamine-labeled cDNA from HAT4-transgenic plants. The single array was scanned successively to detect the fluorescein fluorescence corresponding to mRNA from wild-type plants (C) and the lissamine fluorescence corresponding to mRNA from HAT4-transgenic plants (D). (E and F) A single array was probed with a 1:1 mixture of fluorescein-labeled cDNA from root tissue and lissamine-labeled cDNA from leaf tissue. The single array was then scanned successively to detect the fluorescein fluorescence corresponding to mRNAs expressed in roots (E) and the lissamine fluorescence corresponding to mRNAs expressed in leaves (F).

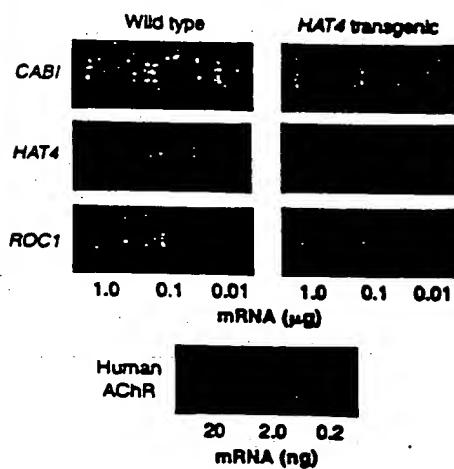


Fig. 2. Gene expression monitored with RNA (Northern) blot analysis. Designated amounts of mRNA from wild-type and HAT4-transgenic plants were spotted onto nylon membranes and probed with the cDNAs indicated. Purified human AChR mRNA was used for calibration.

and D, and Table 2). Hybridization of fluorescein-labeled glucocorticoid receptor cDNA (Fig. 1C) and lissamine-labeled TRP4 cDNA (Fig. 1D) verified the presence of the negative control targets and the lack of optical cross talk between the two fluorophores.

To explore a more complex alteration in expression patterns, we performed a second two-color hybridization experiment with fluorescein- and lissamine-labeled probes prepared from root and leaf mRNA, respectively. The scanning sensitivities for the two fluorophores were normalized by matching the signals resulting from AChR

mRNA, which was added to both cDNA synthesis reactions at a dilution of 1:1000 (Fig. 1, E and F). A comparison of the scans revealed widespread differences in gene expression between root and leaf tissue (Fig. 1, E and F). The mRNA from the light-regulated *CAB1* gene was ~500-fold more abundant in leaf (Fig. 1F) than in root tissue (Fig. 1E). The expression of 26 other genes differed between root and leaf tissue by more than a factor of 5 (Fig. 1, E and F).

The HAT4-transgenic line we examined has elongated hypocotyls, early flowering, poor germination, and altered pigmentation (8). Although changes in expression were

observed for HAT4, large changes in expression were not observed for any of the other 44 genes we examined. This was somewhat surprising, particularly because comparative analysis of leaf and root tissue identified 27 differentially expressed genes. Analysis of an expanded set of genes may be required to identify genes whose expression changes upon HAT4 overexpression; alternatively, a comparison of mRNA populations from specific tissues of wild-type and HAT4-transgenic plants may allow identification of downstream genes.

At the current density of robotic printing, it is feasible to scale up the fabrication process to produce arrays containing 20,000 cDNA targets. At this density, a single array would be sufficient to provide gene-specific targets encompassing nearly the entire repertoire of expressed genes in the *Arabidopsis* genome (2). The availability of 20,274 ESTs from *Arabidopsis* (1, 9) would provide a rich source of templates for such studies.

The estimated 100,000 genes in the human genome (10) exceeds the number of *Arabidopsis* genes by a factor of 5 (2). This modest increase in complexity suggests that similar cDNA microarrays, prepared from the rapidly growing repertoire of human ESTs (1), could be used to determine the expression patterns of tens of thousands of human genes in diverse cell types. Coupling an amplification strategy to the reverse transcription reaction (11) could make it feasible to monitor expression even in minute tissue samples. A wide variety of acute and chronic physiological and pathological conditions might lead to characteristic changes in the patterns of gene expression in peripheral blood cells or other easily sampled tissues. In concert with cDNA microarrays for monitoring complex expression patterns, these tissues might therefore serve as sensitive *in vivo* sensors for clinical diagnosis. Microarrays of cDNAs could thus provide a useful link between human gene sequences and clinical medicine.

**Table 2.** Gene expression monitoring by microarray and RNA blot analyses; tg, HAT4-transgenic. See Table 1 for additional gene information. Expression levels (w/w) were calibrated with the use of known amounts of human AChR mRNA. Values for the microarray were determined from microarray scans (Fig. 1); values for the RNA blot were determined from RNA blots (Fig. 2).

Gene	Expression level (w/w)	
	Microarray	RNA blot
<i>CAB1</i>	1:48	1:83
<i>CAB1</i> (tg)	1:120	1:150
HAT4	1:8300	1:8300
HAT4 (tg)	1:150	1:210
ROC1	1:1200	1:1800
ROC1 (tg)	1:260	1:1300

## REFERENCES AND NOTES

rent EST database (dbEST release 091495) e National Center for Biotechnology Information, Bethesda, MD) contains a total of 322,225 entries, including 255,645 from the human genome. 644 from Arabidopsis. Access is available via the Wide Web (<http://www.ncbi.nlm.nih.gov>). Meyerowitz and R. E. Pruitt. *Science* 229, 1214 (1986); I. Hwang et al., *Plant J.* 1, 367 (1991); S. et al., *Plant Mol. Biol.* 24, 685 (1994); L. Le et al., *Mol. Gen. Genet.* 245, 390 (1994). Ph.D. thesis, Stanford University (1995); — O. Brown, in preparation. Microarrays were made on poly-L-lysine-coated microscope slides (Sigma) with a custom-built arraying machine with one printing tip. The tips loaded 1  $\mu$ l of PCR product (0.5 mg/ml) from 96-well microtiter plates (positively -0.005  $\mu$ m per slide on 40 slides at a size of 500  $\mu$ m. The printed slides were rehydrated 2 hours in a humid chamber, snap-frozen at -70°C for 1 min, rinsed in 0.1% SDS, and treated with 0.5% succinyl anhydride prepared in buffer containing 50% 1-methyl-2-pyrrolidinone and 50% acetic acid. The cDNA on the slides was denatured in distilled water for 2 min at 90°C immediately before use. Microarrays were scanned with a laser scanner that contained a computer-controlled stage and a microscope objective. A mixed helium-laser allowed sequential excitation of fluorophores. Emitted light was split according to wavelength and detected with two photomultipliers. Signals were read into a PC with the use of a bit analog-to-digital board. Additional details on array fabrication and use may be obtained by e-mail ([pbrown@cmgm.stanford.edu](mailto:pbrown@cmgm.stanford.edu)). Usubel et al., Eds., *Current Protocols in Molecular Biology* (Greene & Wiley Interscience, New York, 1994), pp. 4.3.1–4.3.4.

Synthesized [poly(A)]<sup>n</sup> mRNA was prepared from RNA with the use of Oligotex-dT resin (Qiagen). Transcription (RT) reactions were carried out with the use of the Stratascript RT-PCR kit (Stratagene) modified as follows: 50- $\mu$ l reactions contained 0.1  $\mu$ g/ $\mu$ l of pBS mRNA, 0.1 ng/ $\mu$ l of human AChR promoter, 0.05  $\mu$ g/ $\mu$ l of oligo(dT) (21-mer), 1× first-strand buffer, 0.03 U/ $\mu$ l of ribonuclease block, 500  $\mu$ M cytidine triphosphate (dATP), 500  $\mu$ M adenosine triphosphate, 500  $\mu$ M dGTP, 40  $\mu$ M thymidine triphosphate (dTTP), 40  $\mu$ M fluorescein-12-dCTP (or lissamine-5-dCTP), and 0.03 U Stratascript reverse transcriptase. Reactions were incubated for 60 min at 37°C, precipitated with ethanol, and resuspended in 10  $\mu$ l of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Samples were then heated for 3 min at 94°C and chilled on ice. The RNA was purified by adding 0.25  $\mu$ l of 10 N NaOH and by a 10-min incubation at 37°C. The samples were neutralized by addition of 2.5  $\mu$ l of 1 M Tris-HCl (pH 8.0) and 0.25  $\mu$ l of 10 N HCl and precipitated with ethanol. Pellets were washed with 70% ethanol to completion in a Speedvac, resuspended in 10  $\mu$ l of H<sub>2</sub>O, and reduced to 3.0  $\mu$ l in a 30- $\mu$ l volume. Fluorescent nucleotide analogs were obtained from New England Nuclear (DuPont).

Hybridization reactions contained 1.0  $\mu$ l of fluorescent synthesis product (5) and 1.0  $\mu$ l of hybridization mix (10× saline sodium citrate (SSC) and 0.2% SDS). 10× SSC and 0.2% SDS. 2.0- $\mu$ l probe mixtures were aliquoted onto array surface and covered with cover slips (round). Arrays were transferred to a hybridization chamber (3) and incubated for 18 hours at 65°C. Slides were washed for 5 min at room temperature (25°C) in low-stringency wash buffer (1× SSC and 0.1% SDS), then for 10 min at room temperature in high-stringency wash buffer (0.1× SSC and 0.1% SDS). Slides were scanned in 0.1× SSC with the use of a fluorescence laser-scanning device (3).

[poly(A)]<sup>n</sup> mRNA (4, 5) were spotted onto membranes (Nytran) and crosslinked with ultraviolet light with the use of a Stratalinker 1800 (Stratagene). Probes were prepared by random labeling with the use of a Prime-It II kit (Stratagene) in the presence of [<sup>32</sup>P]dATP. Hybridizations were carried out according to the instructions of the manufacturer. Quantitation was performed on a PhosphorImager (Molecular Dynamics).

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12. The laser fluorescent scanner was designed and fabricated in collaboration with S. Smith of Stanford University. Scanner and analysis software was developed by R. X. Xia. The succinic anhydride reaction was suggested by J. Mulligan and J. Van Ness of Dawn Molecular Corporation. Thanks to S. Theodosius, C. Somervelle, K. Yamamoto, and members of the laboratories of R.W.D. and P.O.B. for critical comments. Supported by the Howard Hughes Medical Institute and by grants from NIH (R21HG00450) (P.O.B.) and R37AG00196 (R.W.D.) and by an NSF graduate fellowship (D.S.). P.O.B. is an assistant investigator of the Howard Hughes Medical Institute.

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## Gene Therapy in Peripheral Blood Lymphocytes and Bone Marrow for ADA<sup>-</sup> Immunodeficient Patients

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Adenosine deaminase (ADA) deficiency results in severe combined immunodeficiency, the first genetic disorder treated by gene therapy. Two different retroviral vectors were used to transfer ex vivo the human ADA minigene into bone marrow cells and peripheral blood lymphocytes from two patients undergoing exogenous enzyme replacement therapy. After 2 years of treatment, long-term survival of T and B lymphocytes, marrow cells, and granulocytes expressing the transferred ADA gene was demonstrated and resulted in normalization of the immune repertoire and restoration of cellular and humoral immunity. After discontinuation of treatment, T lymphocytes, derived from transduced peripheral blood lymphocytes, were progressively replaced by marrow-derived T cells in both patients. These results indicate successful gene transfer into long-lasting progenitor cells, producing a functional multilineage progeny.

Severe combined immunodeficiency associated with inherited deficiency of ADA (1) is usually fatal unless affected children are kept in protective isolation or the immune system is reconstituted by bone marrow transplantation from a human leukocyte antigen (HLA)-identical sibling donor (2). This is the therapy of choice, although it is available only for a minority of patients. In recent years, other forms of therapy have been developed, including transplants from haploidentical donors (3, 4), exogenous enzyme replacement (5), and somatic-cell gene therapy (6–9).

We previously reported a preclinical model in which ADA gene transfer and expression

successfully restored immune functions in human ADA-deficient (ADA<sup>-</sup>) peripheral blood lymphocytes (PBLs) in immunodeficient mice *in vivo* (10, 11). On the basis of these preclinical results, the clinical application of gene therapy for the treatment of ADA<sup>-</sup> SCID (severe combined immunodeficiency disease) patients who previously failed exogenous enzyme replacement therapy was approved by our Institutional Ethical Committees and by the Italian National Committee for Bioethics (12). In addition to evaluating the safety and efficacy of the gene therapy procedure, the aim of the study was to define the relative role of PBLs and hematopoietic stem cells in the long-term reconstitution of immune functions after retroviral vector-mediated ADA gene transfer. For this purpose, two structurally identical vectors expressing the human ADA complementary DNA (cDNA), distinguishable by the presence of alternative restriction sites in a nonfunctional region of the viral long-terminal repeat (LTR), were used to transduce PBLs and bone marrow (BM) cells independently. This procedure allowed identification of the origin of

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